Attorney Docket No. 5718-34

POLY ADP-RIBOSE POLYMERASE GENE AND ITS USES

CROSS-REFERENCE

This application claims the benefit of U.S. Provisional Application No. 60/072,785, filed January 27, 1998.

FIELD OF THE INVENTION

The invention is drawn to the genetic manipulation of plants.

BACKGROUND OF THE INVENTION

The physiological and metabolic state of plant cells directly influences the plant response to external stimuli. The plant response to disease includes a host of cellular processes to enable plants to defend themselves from pathogenic agents. These processes apparently form an integrated set of resistance mechanisms that is activated by initial infection and then limits further spread of the invading pathogenic microorganism.

The transformation of plants is a complex process. The process involves contacting cells with a DNA to be integrated into the plant cell genome. Generally, genetic transformation of eukaryotic cells is a random event. That is, the foreign DNA is integrated into the genome at random positions. Often several copies, or parts of copies, of the transforming DNA are integrated in a single position, and/or at different positions, resulting in a transformed cell containing multiple copies of the foreign DNA.

Because the metabolic state of the plant cell is instrumental in various processes, it would be beneficial to be able to influence the state of the cells. Accordingly, there is a need for genes and methods for altering the metabolic state of plant cells.

SUMMARY OF THE INVENTION

Compositions and methods for influencing the metabolic state of plant cells are provided. The compositions comprise poly ADP-ribose polymerase genes and fragments thereof, particularly the maize poly ADP-ribose polymerase gene. The genes or antisense constructions of the genes can be used to transform plant cells and alter the metabolic state of the transformed cell.

In this manner, transformed plants can be obtained having altered metabolic states. The invention has implications in enhancing disease resistance in plants and for methods of genetic transformation of plants.

DETAILED DESCRIPTION OF THE INVENTION

Poly ADP-Ribose Polymerase genes and methods for their use are provided. In particular, the amino acid and nucleotide sequences for the maize poly ADP-ribose polymerase (PARP) are provided as SEQ ID NOs. 2 and 1, respectively. Also of interest are portions of the sequences of the invention. The nucleotide and amino acid sequences of the C-terminal domain of the maize poly ADP-ribose polymerase is provided in SEQ ID NOs. 3 and 4, respectively. The nucleotide sequence of the Zinc fingers is provided in SEQ ID NO. 5.

PARP is generally described as a nuclear enzyme found in most eukaryotes. Structure-function studies have shown that animal PARPs may be divided into at least three subdomains. The N-terminal part contains two zinc fingers and has a high affinity for nicked V-shaped DNA. Interaction of PARP with nicked DNA strongly enhances the activity of the catalytic domain, which is very well conserved among PARPs and located in the carboxyl-terminus of the protein. (Ueda *et al.* (1985) *Ann. Rev. Biochem. 54*:73-100; Sdhah *et al.* (1995) *Anal. Biochem. 227*:1-13).

PARP catalyzes both the transfer of ADP-ribose from NAD^+, mainly to the carboxyl group of a glutamic acid residue on target proteins, and subsequent ADP-ribose polymerization. (Ueda *et al.* (1985) *Ann. Rev. Biochem. 54*:73-100; Sdhah *et al.* (1995) *Anal. Biochem. 227*:1-13)

PARP is generally required in most cases where DNA is cleaved and rejoined, such as in DNA repair, DNA recombination, gene rearrangements and transposition. PARP has been shown to modify PARP itself, histones, high mobility group chromosomal proteins, topoisomerase, endonucleases and DNA polymerases. (Ueda *et al.* (1985) *Ann. Rev. Biochem. 54*:73-100; Sdhah *et al.* (1995) *Anal. Biochem. 227*:1-13)

Initially, the enzyme synthesizes an ester linkage preferentially between the glutamyl(-) or sometimes the C-terminal(-)carboxyl group on the acceptor protein and the 1'-0H of the ribosyly group of ADP-ribose. Subsequently, up to 45-50 ADP units are added via a 2'-1'phosphodiester bond. Branching of the poly (ADP)-ribosyl chains via the 2'-1'phosphodiester linkages is also observed. See, for example, Ueda *et al.* (1985) *Ann. Rev. Biochem. 54*: 73-100; and Shah *et al.* (1995) *Anal. Biochem. 227*:1-13.

Compositions of the invention include isolated nucleic acid molecules encoding the PARP proteins of the invention, as well as fragments and variants thereof. The term "isolated" refers to material, such as a nucleic acid or a protein, which is: (1) substantially or essentially free from components that normally accompany or interact with it as found in its naturally occurring environment. Thus, for a nucleic acid, the sequence is lacking a flanking sequence either 3' or 5' or both. The isolated material optionally comprises material not found with the material in its natural environment; or (2) if the material is in its natural environment, the material has been synthetically (non-naturally) altered by deliberate human intervention to a composition and/or placed at a locus in the cell (e.g., genome or subcellular organelle) not native to a material found in that environment. The alteration to yield the synthetic material can be performed on the material within or removed from its natural state. For example, a naturally occurring nucleic acid becomes an isolated nucleic acid if it is altered, or if it is transcribed from DNA which has been altered, by non-natural, synthetic (i.e., "man-made") methods performed within the cell from which it originates. See, e.g., Compounds and Methods for Site Directed Mutagenesis in Eukaryotic Cells, Kmiec, U.S. Patent No. 5,565,350; *In Vivo* Homologous Sequence

Targeting in Eukaryotic Cells; Zarling *et al.*, PCT/US93/03868. Likewise, a naturally occurring nucleic acid (e.g., a promoter) becomes isolated if it is introduced by non-naturally occurring means to a locus of the genome not native to that nucleic acid. Nucleic acids which are "isolated" as defined herein, are also referred to as "heterologous" nucleic acids.

As used herein, "localized within the chromosomal region defined by and including" with respect to particular markers includes reference to a contiguous length of a chromosome delimited by and including the stated markers.

As used herein, "marker" includes reference to a locus on a chromosome that serves to identify a unique position on the chromosome. A "polymorphic marker" includes reference to a marker which appears in multiple forms (alleles) such that different forms of the marker, when they are present in a homologous pair, allow transmission of each of the chromosomes in that pair to be followed. A genotype may be defined by use of one or a plurality of markers.

As used herein "operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

The nucleotide sequences of the invention can be used to isolate other homologous sequences in other plant species. Methods are readily available in the art for the hybridization of nucleic acid sequences. Coding sequences from other plants may be isolated according to well known techniques based on their sequence homology to the coding sequences set forth herein. In these techniques all or part of the maize coding sequence is used as a probe which selectively hybridizes to other PARP coding sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e. genomic or cDNA libraries) from a chosen organism. For example, the entire maize PARP sequence or portions thereof may be used as probes capable of specifically hybridizing to corresponding coding sequences and messenger

RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify the PARP coding sequences of interest from a chosen organism by the well-known process of polymerase chain reaction (PCR). This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism.

Such techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, *e.g.*. Sambrook *et al., Molecular Cloning*, eds., Cold Spring Harbor Laboratory Press (1989)) and amplification by PCR using oligonucleotide primers corresponding to sequence domains conserved among the amino acid sequences (see, *e.g.* Innis *et al.*, *PCR Protocols, a Guide to Methods and Applications*, eds., Academic Press (1990)). For example, hybridization of such sequences may be carried out under conditions of reduced stringency, medium stringency or even stringent conditions (*e.g.*, conditions represented by a wash stringency of 35-40% Formamide with 5x Denhardt's solution, 0.5% SDS and 1x SSPE at 37 C; conditions represented by a wash stringency of 40-45% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at 42 C; and conditions represented by a wash stringency of 50% Formamide with 5x Denhardt's solution, 0.5% SDS and 1x SSPE at 42 C, respectively), to DNA encoding the PARP genes disclosed herein in a standard hybridization assay. See J. Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual 2d ed.* (1989) Cold Spring Harbor Laboratory. In general, sequences which code for the defense activators and other activator proteins of the invention and hybridize to the sequences disclosed herein will be at least 50% homologous, 70% homologous, and even 85% homologous or more with the disclosed sequence. That is, the sequence similarity of sequences may range, sharing at least about 50%, about 70%, and even about 85% sequence similarity.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b)

"comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" means includes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math. 2*:482; by the homology alignment algorithm of Needleman *et al.* (1970) *J. Mol. Biol. 48*:443; by the search for similarity method of Pearson *et al.* (1988) *Proc. Natl. Acad. Sci. 85*:2444; by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California, GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins *et al.* (1988) *Gene 73*:237-244; Higgins *et al.* (1989) *CABIOS 5*:151-153; Corpet *et al.* (1988) *Nucleic Acids Research 16*:10881-90; Huang *et al.* (1992) *Computer Applications in the Biosciences 8*:155-65, and Person *et al.* (1994) *Methods of Molecular Biology 24*:307-331; preferred computer alignment methods also include the BLASTP, BLASTN, and

BLASTX algorithms. Altschul *et al.* (1990) *J. Mol. Biol. 215*:403-410. Alignment is also often performed by inspection and manual alignment.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences which differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, *e.g.*, as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched

positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

(e) (i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%. Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5 C to about 20 C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent wash conditions are those in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least about 50, 55, or 60 C. However, nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This may occur, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e) (ii) The terms "substantial identity" in the context of peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference

sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman *et al.* (1970) *J. Mol. Biol. 48*:443. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein and hence confer resistance to nematodes. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the entire nucleotide sequence encoding the proteins of the invention.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the proteins conferring resistance to nematodes. Generally, nucleotide sequence variants of the invention will have at least 70%, generally, 80%, preferably up to 90% sequence identity to its respective native nucleotide sequence.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Methods for

such manipulations are generally known in the art.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the activator proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel *et al.* (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; U.S. Patent No. 4,873,192; Walker and Gaastra (eds.) *Techniques in Molecular Biology*, MacMillan Publishing Company, NY (1983) and the references cited therein. Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as variant and mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variants and modified forms thereof. Such variants will continue to possess the desired PARP activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

PARP is present in all higher eukaryotes. Therefore, it is recognized that the nucleotide sequence encoding the PARP may be utilized from any eukaryotic source, including vertebrates, arthropods, mollusks, slime moulds, dinoflagellates, fungi, mammals, chicken, Xenopus and insects. See, for example, Heller *et al.* (1995) *J. Biol. Chem. 270*:11178-11180; Schreiber *et al.* (1995) *Proc. Natl. Acad. Sci. USA 92*:4753-4757; Ueda *et al.* (1985) *Ann. Rev. Biochem. 54*:73-100; Brightwell *et al.* (1975) *Biochem. J. 147*:119-129; Kofler *et al.* (1993) *ibid 293*:275-281; Collinge *et al.* (1994) *Mol. Gen. Genet. 245*:686-693; Scovassi *et al.* (1986) *Eur. J. Biochem. 159*:77-84; Simonin *et al.* (1991) *Anal. Biochem. 195*:226-231; Masutani *et al.* (1994) *Eur. J. Biochem. 220*:607-614; herein incorporated by reference.

It is recognized that the plant cell can be transformed with a nucleotide sequence encoding PARP, a nucleotide sequence encoding a portion of PARP, preferably the C-terminal portion of PARP, as well as with a nucleotide sequence

encoding the antisense sequence for the PARP gene, or portions thereof. In this manner, the level of expression of the PARP in the plant cell can be modulated, i.e., increased or decreased, respectively. Levels of expression of the sense or antisense sequence can be regulated by the promoter utilized to express the gene.

Promoters for the expression of genes in plant cells are known in the art. Promoters are available for constitutive, tissue specific, inducible, etc. Such promoters include, for example, 35S promoter, Meyer *et al.* (1997) *J. Gen. Virol. 78*:3147-3151; biotin carboxylase, Bas *et al.* (1997) *Plant Mol. Biol. 35*:539-550; oxidase, Lasserre *et al.* (1997) *Mol. Gen. Genet 256*:211-222; cab, Shiina *et al.* (1997) *Plant Physiol. 115*:477-483; phospholipase, Xu *et al.* (1997) *Plant Physiol. 115*:387-395; farnesyltransferase, Zhou *et al.* (1997) *Plant J. 12*:921-930; plastocyanin, Helliwell *et al.* (1997) *Plant J. 12*:499-506; CVMV promoter, Verdaquer *et al.* (1996) *Plant Mol. Biol. 31*:1129-1139; actin, An *et al.* (1996) *Plant J. 10*:107-121; heat shock, Prandl *et al.* (1996) *Plant Mol. Biol. 31*:157-162; ubiquitin, thionin, 35S, Holtorf *et al.* (1995) *Plant Mol. Biol. 29*:637-646; Callis *et al.* (1990) *J. Biol. Chem. 265*:12486-12493; histone, Atanossova *et al.* (1992) *Plant J. 2*:291-300; rol C, Fladung *et al.* (1993) *Plant Mol. Biol. 23*:749-757; histone, Brignon *et al.* (1993) *Plant J. 4*:445-457; Lepetit *et al.* (1992) *Mol. Gen. Genet. 231*:276-285.

As indicated, recent studies on the mechanism of PARP suggests involvement of the enzyme in regulation of DNA repair, recombination and replication. The enzyme is rapidly activated by DNA and exhibits a high affinity for naked single-stranded or double-stranded DNA. Any perturbation in the cellular morphology and/or physiology that causes a change in chromatin conformation generally results in a rapid increase in PARP activity. PARP is an important modulator of the fate of DNA introduced into a plant cell. Accordingly, plants transformed with either a sense or antisense PARP nucleotide sequence may be utilized to increase transformation frequency in plant cells. Therefore, the present invention provides for the regulation of the levels of PARP in the plant cell to determine its effect on plant transformation and gene targeting.

It is further recognized that because the enzyme plays a role in cellular stress, it may be beneficial to increase the levels of the enzyme to prevent plant disease or pathogen attack. In this manner, constitutive or inducible promoters may be utilized. Constitutive promoters include, for example, those disclosed in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142, herein incorporated by reference. Inducible promoters are known in the art and include, for example, pathogen inducible promoters, such as promoters from pathogenesis-related proteins (PR proteins) which are induced following infection by a pathogen; *e.g.*, PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol. 89*:245-254; Uknes *et al.* (1992) *The Plant Cell 4*:645-656; and Van Loon (1985) *Plant Mol. Virol. 4*:111-116. Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol. 9*:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions 2*:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:2427-2430; Somsisch *et al.* (1988) *Molecular and General Genetics 2*:93-98; and Yang, Y (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:2507-2511; Warner *et al.* (1993) *Plant J. 3*:191-201; Siebertz *et al.* (1989) *Plant Cell 1*:961-968; and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiological and Molecular Plant Pathology 41*:189-200).

The PARP genes or antisense nucleotides of the invention can be introduced into any plant. The genes or nucleotide sequences to be introduced will be used in expression cassettes for expression in any plant of interest.

Such expression cassettes will comprise a transcriptional initiation region linked to the gene encoding the PARP gene or antisense nucleotide of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By foreign is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

The transcriptional cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot (1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-158; Ballas *et al.* (1989) *Nucleic Acids Res. 17*:7891-7903; Joshi *et al.* (1987) *Nucleic Acid Res. 15*:9627-9639.

The genes of the invention are provided in expression cassettes for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to the gene of interest. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on another expression cassette. Where appropriate, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant preferred codons for improved expression. Methods are available in the art for synthesizing plant preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436, 391, and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious

polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences which may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *PNAS USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak *et al.* (1991) *Nature 353*:90-94; untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling *et al.* (1987) *Nature 325*:622-625; tobacco mosaic virus leader (TMV), (Gallie, D.R. *et al.* (1989) *Molecular Biology of RNA*, pages 237-256; and maize chlorotic mottle virus leader (MCMV) (Lommel, S.A. *et al.* (1991) *Virology 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiology 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.* transitions and transversions, may be involved.

The genes of the present invention can be used to transform any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be

obtained. Transformation protocols may vary depending on the type of plant or plant cell, *i.e.* monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) Biotechniques *4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* *83*:5602-5606, *Agrobacterium* mediated transformation (Hinchee *et al.* (1988) *Biotechnology 6*:915-921), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; Tomes *et al.* Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment In Gamborg and Phillips (eds.) *Plant Cell, Tissue and Organ Culture: Fundamental Methods*, Springer-Verlag, Berlin (1995); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see, Weissinger *et al.* (1988) *Ann. Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes *et al.* Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment in Gamborg and Phillips (eds.) *Plant Cell, Tissue and Organ Culture: Fundamental Methods*, Springer-Verlag, Berlin (1995) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooydaas-Van Slogteren & Hooykaas (1984) *Nature (London) 311*:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues* ed. G.P. Chapman *et al.*, pp. 197-209. Longman, NY (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418; and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

The present invention also provides isolated nucleic acid comprising polynucleotides of sufficient length and complementarity to a gene to use as probes or amplification primers in the detection, quantitation, or isolation of gene transcripts. For example, isolated nucleic acids of the present invention can be used as probes in detecting deficiencies in the level of mRNA in screenings for desired transgenic plants, for detecting mutations in the gene (e.g., substitutions, deletions, or additions), for monitoring upregulation of expression or changes in enzyme activity in screening assays of compounds, for detection of any number of allelic variants (polymorphisms) of the gene, or for use as molecular markers in plant breeding programs. The isolated nucleic acids of the present invention can also be employed for use in sense or antisense suppression of a PARP gene in a host cell, tissue, or plant. See, *Tools to Determine the Function of Genes*, 1995 Proceedings of the Fiftieth Annual Corn and Sorghum Industry Research Conference, American Seed Trade Association, Washington, D.C., 1995. Additionally, non-translated 5 or 3 regions of the polynucleotides of the present invention can be used to modulate turnover of heterologous mRNAs and/or protein synthesis.

The present invention provides a method of genotyping a plant comprising a polynucleotide of the present invention. Preferably, the plant is a monocot, such as maize or sorghum. Genotyping provides a means of distinguishing homologs of a chromosome pair and can be used to differentiate segregants in a plant population. Molecular marker methods can be used for phylogenetic studies, characterizing genetic relationships among crop varieties, identifying crosses or somatic hybrids,

localizing chromosomal segments affecting monogenic traits, map based cloning, and the study of quantitative inheritance. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Chapter 7, Clark, Ed., Springer-Verlag, Berlin (1997). For molecular marker methods, see generally, The DNA Revolution by Andrew H. Paterson 1996 (Chapter 2) in: Genome Mapping in Plants (ed. Andrew H. Paterson) by Academic Press/R. G. Landis Company, Austin, Texas, pp.7-21.

The particular method of genotyping in the present invention may employ any number of molecular marker analytic techniques such as, but not limited to, restriction fragment length polymorphisms (RFLPs). RFLPs are the product of allelic differences between DNA restriction fragments caused by nucleotide sequence variability. As is well known to those of skill in the art, RFLPs are typically detected by extraction of genomic DNA and digestion with a restriction enzyme. Generally, the resulting fragments are separated according to size and hybridized with a probe; single copy probes are preferred. Restriction fragments from homologous chromosomes are revealed. Differences in fragment size among alleles represent an RFLP. Thus, the present invention further provides a means to follow segregation of a PARP gene or nucleic acid of the present invention as well as chromosomal sequences genetically linked to these genes or nucleic acids using such techniques as RFLP analysis. Linked chromosomal sequences are within 50 centiMorgans (cM), often within 40 or 30 cM, preferably within 20 or 10 cM, more preferably within 5, 3, 2, or 1 cM of a PARP gene.

In the present invention, the nucleic acid probes employed for molecular marker mapping of plant nuclear genomes selectively hybridize, under selective hybridization conditions, to a gene encoding a polynucleotide of the present invention. In preferred embodiments, the probes are selected from polynucleotides of the present invention. Typically, these probes are cDNA probes or *Pst I* genomic clones. The length of the probes is discussed in greater detail, *supra*, but are typically at least 15 bases in length, more preferably at least 20, 25, 30, 35, 40, or 50 bases in length. Generally, however, the probes are less than about 1 kilobase in length. Preferably, the probes are single copy probes that hybridize to a unique locus in a haploid

chromosome complement.

The method of detecting an RFLP comprises the steps of (a) digesting genomic DNA of a plant with a restriction enzyme; (b) hybridizing a nucleic acid probe, under selective hybridization conditions, to a sequence of a polynucleotide of the present of said genomic DNA; (c) detecting therefrom a RFLP. Other methods of differentiating polymorphic (allelic) variants of polynucleotides of the present invention can be had by utilizing molecular marker techniques well known to those of skill in the art including such techniques as: 1) single stranded conformation analysis (SSCP); 2) denaturing gradient gel electrophoresis (DGGE); 3) RNase protection assays; 4) allele-specific oligonucleotides (ASOs); 5) the use of proteins which recognize nucleotide mismatches, such as the *E. coli* mutS protein; and 6) allele-specific PCR. Other approaches based on the detection of mismatches between the two complementary DNA strands include clamped denaturing gel electrophoresis (CDGE); heteroduplex analysis (HA); and chemical mismatch cleavage (CMC). Thus, the present invention further provides a method of genotyping comprising the steps of contacting, under stringent hybridization conditions, a sample suspected of comprising a polynucleotide of the present invention with a nucleic acid probe. Generally, the sample is a plant sample; preferably, a sample suspected of comprising a maize polynucleotide of the present invention (e.g., gene, mRNA). The nucleic acid probe selectively hybridizes, under stringent conditions, to a subsequence of a polynucleotide of the present invention comprising a polymorphic marker. Selective hybridization of the nucleic acid probe to the polymorphic marker nucleic acid sequence yields a hybridization complex. Detection of the hybridization complex indicates the presence of that polymorphic marker in the sample. In preferred embodiments, the nucleic acid probe comprises a polynucleotide of the present invention.

EXPERIMENTAL

Materials and Methods

Chemicals and Reagents

All chemicals used in this study were of molecular biology grade. Trizma base (Tris (hydroxymethyl) aminomethane; abbreviated hereafter as Tris), N-2 hydroxy-ethyl-pipcrazine-N'-2-ethane sulfonic acid (Hepes), Ethylenediaminetetraacetic acid, sodium salt (EDTA), Magnesium chloride, Urea, were procured from Sigma Chemical Co. Analytical grade glycerol was obtained from Baxter. Dithiothreitol (DTT), PefablocSC, Pepstatin, Bestatin, all restriction enzymes, DNA and RNA purification kits as well as markers were purchased from Boehringer Mannheim. Immunodetection kits for Western blots, silver staining and Colloidal Coomassie Blue staining were from Novex. All radioactive chemicals were purchased from NEN-Dupont and NEN. Chromotopographic resins were purchased either from Sigma, BioRad or Pharmacia.

Cell Culture

The enzyme is isolated from a Hi II embryogenic callus cell line. Exponentially growing cultures of 612B4 cells were maintained in dark at 28°C. (Armstrong *et al.* (1992) *Theor. Appl. Genet. 84*:755-762). The cell suspensions are in MS medium supplemented with 2-4-dichlorophenoxyaxcctic acid (2.5 mg/l). Cultures are grown for a week on a gyroratatory shaker at 150 rpm and harvested by decantation. Routinely, 60-80 g of cells are obtained from 800-900 ml cultures grown in 12-14 flasks.

Cells are harvested by filtration and used to prepare whole cell extracts (WCE) form 612B4 cells using the Bionebulizer (Glas-Col, Terre Haute, Indiana). The process for WCE preparation is outlined in Schema 1. All operations were carried out at 4°C or on ice unless mentioned otherwise.

Chromatography on Heparin-agarose: About 300 ml of Heparin -agarose (Sigma) was washed extensively with 20 mM Hepes-KOH pH 7.9, 0.1 mM EDTA,

20% glycerol (HGED buffer), packed into a 5.0 x 30 cm Econo column (Biorad) and connected to the Econo System (Biorad). The matrix was equilibrated with HGED + 100 mM KCI. Three batches of crude WCE extract (approx. 1.8-2.0 g of total pooled protein in 60-80 ml) were loaded on the column at a flow rate of 15-20 ml/hr. The column was washed extensively with equilibration buffer till the A_{280} of the effluent was < 0.1 unit (approx. 900 ml).

Small aliquots of peak fractions were saved for PARP assays and all fractions (7-8 ml each) showing A_{280} > 0.1 unit were pooled. Protein was precipitated by adding solid ammonium sulfate (0.4 g/ml). The mixture was centrifuged at 40,000 x g for 30 min., dissolved in minimum amount HGED and dialyzed against HGED+100 mM KCI containing Pefabloc and DTT. This fraction is designated HA-1. The column was further washed with 900 ml each HGED + 400 mM KCI followed by HGED + 1 M KCI. Fractions from both washes were processed as above and designated as HA-2 and HA-3. PARP assays were performed on HA-1, 2 and 3 and the active fraction (HA-2) was used for further purification.

Chromatography on DNA-cellulose: DNA-cellulose (Sigma) was washed extensively with HGED and packed in the Econo column (2.5 x 30 cm). The column was connected to the Econo System and equilibrated with HGED+100mM KCI. Partially pure PARP from three Heparin-agarose column was loaded on the DNA-cellulose column. Unbound protein was removed by washing with HGED + 100 mM KCI (200 ml; designated as DC-1). The bound protein was eluted with HGED + 1M KCI (designated DC-2). All fractions was processed as described above for activity and protein.

Chromatography on Histone-agarose: Histone-agarose (Sigma) was washed extensively with HGED and packed into an Econo column (1.5 x 15 cm). The column was equilibrated in HGED + 100 mM KCI. Active fraction from DNA-cellulose (DC-2) was further fractionated on Histone-agarose by washing the column successively with HGED containing 100 mM, 400 mM and 1 M KCI. All fractions were processed

as above and dialyzed against 20 mM Tris-HCI buffer pH 7.9 containing 100 mM KCI.

Chromatography on Mini-Q column: Mini-Q column (Pharmacia) was connected to Smart-LC (Pharmacia) and equilibrated by washing with five bed volumes of HGED followed by five bed volumes of Tris-HCI buffer + 100 mM KCI. Active PARP from the Histone-agarose step was loaded on the column. The column was washed with three bed volumes of equilibration buffer and 400 µl fractions were collected. Column was further developed using a step gradient of KCI at 400 mM, 600 mM and 1M in Tris-HCI, pH 8.0. All fractions were tested for PARP activity as described below.

Enzyme Assays

Catalytic activity of PARP is assayed following published protocols (Shah *et al.* (1995) *Anal. Biochem* 227:1-13) with modifications suitable for the plant enzyme. Briefly, the enzyme (in a total volume of 25 µl of 20 mM Hepes pH 7.9, 100 mM KCI) is incubated with 2.5-5 µCi of ∞-^{32}P-NAD+, 2 µl/ml final concentration of bovine histone (fraction IV), 2 µg of activated calf thymus DNA and 0.5 mM DTT. The reactions are carried out at 6°C unless otherwise mentioned. At the end of the appropriate time intervals, the labeled protein is precipitated with 25% TCA. The precipitate is collected by centrifugation at 16,000 x g for 10 min., washed 2 x with 5% TCA and counted in a LSC. Protein heated at 65°C for 5 min. is used as a negative control.

Microsequencing

Protein samples obtained from the Mini-Q column purification step was electrophoresed in duplicate on a 10% polyacrylamide gels using 0.1% SDS in the running buffer (Shah *et al.* (1995) *Anal. Biochem* 227:1-13). One half of the gel was used to detect protein bands with a Colloidal Coormassie staining kit (Novex) following manufacturer s instructions. The other half was used in the activity blot assay to confirm position of the active PARP on the gel. Stained protein band

corresponding to active PARP was cut out from the gel and used microsequencing carried out at the W.M. Keck Foundation Biotechnology Resource Laboratory if Yale University. *In gel* tryptic digestion of the protein, Matrix Assisted Laser Desorption Mass Spectrometry (MALDI-MS) of the isolated peptides, and amino sequencing of representative peptides was carried out following protocols detailed elsewhere (Stone *et al.* (1990) *In: Methods in Enzyomology 193*:389-412); (Stone *et al.* (1991) *In: Methods in Protein Sequence Anal.ysis 133*-141); Williams *et al.* (1995) *In: Techniques in Protein Chemistry 6*:143-152); Williams *et al.* (1995) *In: Protein Protocol Handbook 365*-378).

Antipeptide Antibodies

Synthesis of the peptide antigens and antibody generation was carried out at Research Genetics, Inc. Two peptides (P-1 and P-2) were used as for antibody generation using two different protocols. In the first protocol, peptide P-1 was synthesized as a multiple antigenic peptide (MAP) following published protocols (Tam, J.P. (1988) *Proc. Nat. Acad. Sci. USA 85*:5409-5413). Antiserum was collected and analyzed for cross-reactivity to PARP using Western blots (Shah *et al.* (1995) *Anal. Biochem 227*:1-13). In the second protocol, P-2 was synthesized as MAP (Tam, J.P. (1988) *Proc. Natl. Acad. Sci. USA 85*:5409-5413) as well as a linear peptide (Barany *et al.* (1980) *In: The Peptides 2*:1-284). The linear peptide was conjugated to hemocyanin using published methods (Walter *et al.* (1980) *Proc. Natl. Acad. Sci. USA 77*:5197-5200) and used for immunization. The immunization protocol for both types of antigens was essentially the same and is detailed below.

Two New Zealand rabbits (4-6 months old) were used for immunization with each type of antigen. The antigens were prepared by dissolving 500 µg MAP peptide in 500 µl of saline and mixed with equal 500 µl of complete Freund's adjuvant and injected subcutaneously at three to four dorsal sites. Same concentration of each antigen (in saline) was mixed with equal volume of incomplete Freund's adjuvant and injected as before at two, four and six weeks after the first immunization. Animals were bled from the auricular artery to collect 30-50 ml blood on days 0, 27, 57 and 69.

Blood samples were allowed to clot at room temperature for 15 min. and serum was isolated from each sample by centrifugation at 5,000 x g for 10 min. Cell-free serum was decanted gently into a clean tube and stored at –20°C till further use.

cDNA Cloning

Total RNA was isolated from corn tissues with TRIzol Reagent (Life Technology, Inc. Gaithersburg, MD) using a modification of the guanidine isothiocyanate/acid-phenol procedure described by Chomczynski and Sacchi (Chomczynski *et al.* (1987) *Anal. Biochem 162*:156). In brief, plant tissue samples were pulverized in liquid nitrogen before the addition of the TRIzol Reagent, and then were further homogenized with a mortar and pestle. Addition of chloroform by centrifugation was conducted for separation of an aqueous phase and an organic phase. The total RNA was recovered by precipitation with isopropyl alcohol from the aqueous phase.

The selection of poly(A) + RNA from total RNA was performed using PolyATract system (Promega Corporation, Madison, WI). In brief, biotinylated oligo (dT) primers were used to hybridize to the 3' poly(A) tails on mRNA. The hybrids were captured using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA was washed at high stringent condition and cluted by RNase-free deionized water.

Synthesis of the cDNA was performed and unidirectional cDNA libraries were constructed using the SuperScript Plasmid System (Life Technology, Inc., Gaithersburg, MD). First strand of cDNA was synthesized by priming an oligo(dT) primer containing a Not I site. The reaction was catalyzed by SuperScript Reverse Transcriptase II at 45°C. The second strand of cDNA was labeled with alpha-^{32}P-dCTP and portions of the molecules smaller than 500 base pairs and unligated adapters were removed by Sephacryl-S400 chromatography. The selected cDNA molecules were ligated into pSPORT1 reference vector between the Not I and Sal I sites.

Individual colonies were picked and DNA was prepared either by PCR with M13 forward primers and M13 reverse primers, or by plasmid miniprep isolation. All the cDNA clones were sequenced using M13 reverse primers.

Analytical

Protein was estimated by the Bradford method (Bradford, M. (1976) *ibid* 72:248-254) using bovine γ-globulin as standard. Activity blots, Western blots and product analysis were performed essentially following published protocols (10, 20-22), except that all essays were carried out at 6°C.

Identification of Zinc Fingers

Two PCR primers were designed to encompass both the Zinc fingers of the maize PARP sequence. These primers were used for reverse transcriptase assisted PCR using the Titan 1 tube RT-PCR kit from Boeheringer Mannheim. Maize callus and leaf mRNA was used as template. The PCR product was purified using Qia Quick PCR product purification columns (Qiagen) and sequenced using an ABI sequencer. Sequenced data is shown in SEQ ID NO. 5.

Isolation of PARP from maize cells

Cells
↓
(60-80 g)
↓
Suspend in Nebulization Buffer (4 ml/g)
↓
(20 mM Hepes pH 7.9, 20% glycerol, 0.4 molal sorbitol, 0.1 mM EDTA)
(+DTT and PI cocktail)
↓
Bionebulization at 100 psi x 4
↓
Filter through cheesecloth
↓
Filtrate
↓
Add 0.1 volume of SASS

↓

Mix gently for 1 hr.

↓

Centrifuge at 100,000 x g

↓

Add 0.4 of solid $(NH_4)_2SO_4$ per ml/S100

↓

Mix for 30 min.

↓

Centrifuge at 40,000 x g for 30 min.

↓

Dissolve precipitate in HGED buffer and dialyze overnight

↓

Centrifuge at 40,000 x g for 30 min.

↓

Store WCE at –80°C

Scheme I

ll publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.